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Asymmetrical generalisation between pheromonal and floral odours in appetitive olfactory conditioning of the honey bee (Apis mellifera L.)

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Abstract The capacity to generalise between similar but not identical olfactory stimuli is crucial for honey bees, allowing them to find rewarding food sources with varying volatile emissions. We studied bees’ generalisation behaviour with odours having different biological values: typical floral odours or alarm compounds. Bees’ behavioural and peripheral electrophysiological responses were investigated using a combined proboscis extension response conditioning-electroantennogram assay. Bees were conditioned to pure linalool (floral) or to pure isoamyl acetate (alarm) and were tested with different concentrations of both compounds. Electrophysiological responses were not influenced by conditioning, suggesting that the learning of individual compounds does not rely on modulations of peripheral sensitivity. Behaviourally, generalisation responses of bees conditioned to the alarm compound were much higher than those of bees conditioned to the floral odour. We further demonstrated such asymmetrical generalisation between alarm and floral odours by using differential conditioning procedures. Conditioning to alarm compounds (isoamyl acetate or 2-heptanone) consistently induced more generalisation than conditioning to floral compounds (linalool or phenylacetaldehyde). Interestingly, generalisation between the two alarm compounds, which are otherwise chemically different, was extremely high. These results are discussed in relation to the neural representation of compounds with different biological significance for bees.

Keywords Olfactory learning · Generalisation · Electroantennogram · Proboscis extension response conditioning · Alarm pheromones

Abbreviations CS conditioned stimulus · EAG electroantennogram · GI generalisation index · PER proboscis extension response · US unconditioned stimulus

Introduction

At the individual level, the foraging behaviour of honey bees is based on learning and using various floral stimuli, including odours (Koltermann 1969), colours (Menzel 1967), patterns and shapes (Wehner 1981). Of these cues, olfactory signals play a major role in the recognition of food sources (Kriston 1973; Menzel et al. 1993). Bees are able to differentiate a large number of olfactory signals from their environment (Vareschi 1971; Laska et al. 1999) and learn which predict food rewards and which do not (Menzel et al. 1993). However, under natural conditions honey bees confront floral odours, which are blends containing tens to hundreds of components. The composition of such floral odours varies in quality or in quantity both over time and in space (Pham-Delègue et al. 1989, 1992). Therefore, generalisation, defined as the tendency of animals to respond behaviourally to stimuli which differ from a learnt stimulus (Pearce 1987) is a fundamental process for bees’ survival: it allows bees to find fruitful food sources in spite of fluctuations in those sources’ volatile emissions.

In theory, animals generalise between stimuli because these stimuli activate similar neural representations (Pearce 1987; Shepard 1987). The more the presented stimulus differs from the learnt one, i.e. the more distant representations of stimuli are in the psychological space of animals, the less generalisation is observed. The
study of generalisation is thus a means of understanding how animals perceive the stimuli of their environment. In bees, olfactory generalisation was mainly studied in an appetitive learning context, where workers are trained to associate an odour to a sugar reward. Bees conditioned to individual compounds (‘compound’ being defined as a given chemical entity) or to mixtures were shown to generalise their responses to a wide range of other olfactory stimuli (Smith and Menzel 1989; Smith 1991; Pham-Delégue et al. 1993). In particular, they generalise more between compounds belonging to the same chemical class (i.e. with the same functional group) than between compounds of different classes (Smith and Menzel 1989; Getz and Smith 1990). Within a given chemical class, they seem to generalise more between compounds with similar carbon chain lengths than with dissimilar lengths (Laska et al. 1999). In terms of stimulus intensity, generalisation between two doses of the same compound decreases with the dose difference, and more along decreasing than along increasing concentrations (Pham-Delégue et al. 1993; Bhagavan and Smith 1997). Thus, considerable knowledge about the rules governing olfactory generalisation in bees has been gained in the last decade. Recently, optical imaging studies of neural activity at the level of the glomeruli of the antennal lobe, the primary olfactory neuropile of the bee brain, revealed the essential principles of olfactory coding and a first step toward understanding the neural representation of odours (Joerges et al. 1997; Sachse et al. 1999; Galizia and Menzel 2001). However, no effort has yet been invested in understanding what effect the functional value of different chemical entities, i.e. their biological significance for bees, could have on their neural representation.

Beside floral odours which mediate foraging, other olfactory stimuli play a major role in the social life of bees: pheromones are used for reproduction, social communication and cohesion and the defence of the society (Free 1987). Although floral odours elicit highly plastic behaviours, pheromonal compounds give way to relatively fixed behavioural patterns. The neural processing of pheromonal and food odours is generally believed to be based on two subsystems: one, a very specific system, limited to the recognition of pheromonal compounds (the specialist system) and a second, more broadly tuned, responding to a wide range of food-associated odours (Masson and Mustaparta 1990; Hildebrand and Shepherd 1997). Now, both type of odours can be learnt by honey bees in an appetitive context (Pham and Masson 1985; Marfaing et al. 1989; Getz and Smith 1990). Optical imaging studies of olfactory coding at the level of the antennal lobes led Joerges et al. (1997) to suggest that both kinds of substances differ in their neural coding. Pheromonal odours would yield activation patterns which remain constant among different individuals, whilst floral odours would generate more variable patterns. Galizia et al. (1999) contested this view such that it is unclear whether the neural coding of pheromones and floral odours follows the same principles. In order to improve our understanding of the neural representation of such compounds in honey bees, we compared bees’ learning and generalisation performances with pheromonal odours to those obtained with common floral odours.

To study learning and generalisation in an appetitive context, we used the procedure for conditioning the proboscis extension response (PER) of honey bees (Kuwabara 1957; Bitterman et al. 1983). As differential treatment of pheromonal and floral odours may already take place at the peripheral level, and since previous work showed modulations of peripheral sensitivity during olfactory conditioning with floral odours (De Jong and Pham-Delégue 1991; Wadhams et al. 1994), we combined PER conditioning with electroantennogram (EAG) recordings on live bees. We thus studied the patterns of behavioural and electrophysiological responses during generalisation behaviour with both floral and alarm odours.

**Materials and methods**

**Bees**

Emerging Italian worker bees, *Apis mellifera ligustica*, were collected from outdoor hives. They were caged in groups of about 50 individuals, maintained in an incubator at 33°C, 55% RH, and fed ad libitum with sugar, pollen and water. Fourteen- to sixteen-day-old bees were used in the experiments since workers usually become foragers at this age (Sakagami 1953; Seeley 1982) and give the most consistent performances in the PER conditioning assay (Pham-Delégue et al. 1990). Before the experiments, bees were starved for 2 hours in the cage, and were individually mounted in glass holders, leaving only their antennae and mouth parts free.

**Odour stimuli**

In experiment 1, odour stimuli used for the conditioning procedure were pure +/- linalool (Sigma, 97%) or pure isoamyl acetate (Janssen Chimica, 99%). The former is a constituent of floral odours (Knudsen et al. 1993) whilst the latter is the main component of the sting alarm pheromone of honey bees (Boch et al. 1962). Doses used for conditioning (i.e. contained in 10 µl) were 8.65 mg and 8.76 mg, respectively (Stecher et al. 1968). For the testing procedure, serial dilutions were made up in hexane (Prolabo, 95%) in order to obtain doses applied onto the filter paper (i.e. contained in 10 µl) of 10^{-7} g, 10^{-6} g, 10^{-5} g and 10^{-4} g. The stimuli used for testing were therefore approximately the equivalent of 0.001–1% of the dose used for conditioning. In experiment 2, all odour stimuli were presented in their pure form. Two more compounds were used, phenylacetalddehyde (Sigma, 95%) and 2-heptanone (Sigma, 99.8%). The former is a common floral volatile (Knudsen et al. 1993) and the latter is the unique component of the alarm pheromone produced by the mandibular glands of bees (Shearer and Boch 1965). The doses of these compounds used for conditioning (i.e. contained in 10 µl) were 10.23 mg and 8.20 mg, respectively.

**Behavioural and electrophysiological responses of bees conditioned to a floral or a pheromonal compound (experiment 1)**

Bees were subjected to a PER conditioning procedure with either a pure floral odour (linalool) or a pure alarm compound (isoamyl